3.3 Plasmodium *Sporozoite ELISA*

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Introduction

Enzyme-linked immunosorbent assays (ELISAs) were developed to detect *Plasmodium falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite (CS) proteins in malaria-infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the monoclonal antibodies (Mabs) used. The ELISAs detect CS proteins, which can be present in the developing oocysts, dissolved in haemolymph, and on sporozoites present in the haemocoel or in the salivary glands.

Therefore, a positive ELISA may detect CS in organs other than the salivary glands and does not establish that species as a vector. ELISA results also may not be concordant with detection of sporozoites from salivary gland dissections.

ELISAs can be carried out on fresh, frozen, or dried mosquitoes. If specimens are to be dried, they must be processed quickly and kept dry (stored with desiccant) to prevent microbial growth that can result in high background values. Before collection of the mosquitoes is initiated, consideration should be given to the possibility of conducting other tests (e.g., molecular, host blood meal, etc.) that may require different storage conditions or extraction buffers. Voucher specimens should also be collected and saved.

The "sandwich" ELISA is begun by adsorption of the capture Mab to the wells of a microtiter plate (**Figure 3.3.1**). After the capture Mab has bound to the plate, the well contents are aspirated and the remaining binding sites are blocked with blocking buffer. Mosquitoes to be tested are ground in blocking buffer containing IGEPAL CA-630, and an aliquot is tested. Positive and negative controls are also added to specific plate wells at this time. If CS antigen is present (depicted as diamond in Fig. 1.B) it will form an antigen-antibody complex with the capture Mab. After a 2-hour incubation at room temperature, the mosquito homogenate is aspirated and the wells are washed. Peroxidase-linked Mab is then added to the wells, completing the formation of the "sandwich" (Fig. 1.C). After 1 hour, the well contents are aspirated, the plate is washed again and the clear peroxidase substrate solution is added (Fig 1.D). As the peroxidase enzyme reacts with the substrate, a dark green product is formed (Fig 1.D), the intensity of the color is proportional to the amount of CS antigen present in the test sample.

Results are read visually or at 405-414 nm using an ELISA plate reader 30 and/or 60 minutes after the substrate has been added. ELISA positive mosquitoes should be retested to: a) confirm positives and b) estimate the amount of CS protein per mosquito if desired.

Because the volume of mosquito homogenate is not sufficient for numerous tests, users may choose to ‘recycle’ the sample by transfering the homogenate to a second plate containing a different antibody.

For technical advice or recommendations regarding the use of this protocol, please contact:

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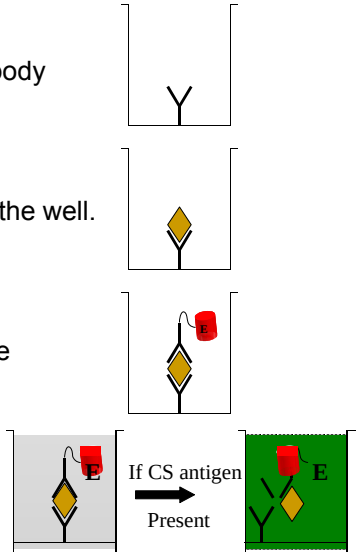
Figure 3.3.1. The "sandwich" ELISA for detection of *Plasmodium falciparum* and *P. vivax* circumsporozoite proteins.

A. Anti-sporozoite monoclonal antibody adsorbed to the plate.

B. Mosquito homogenate added to the well.

C. Peroxidase-linked anti-sporozoite monoclonal antibody added.

D. ABTS substrate added.



Preparation of Sporozoite ELISA Solutions

The following solutions should be prepared prior to performing the procedure. Do not add sodium azide to solutions as it is a peroxidase inhibitor. We also no longer add thimerosal to the solutions, as this is mercury-based and presents problems with proper disposal. Keep all solutions in the refrigerator when not in use and adhere to shelf lives to prevent problems related to microbial growth in working solutions.

Order supplies from the suggested vendors or insure that they are identical to those recommended. Be aware too that different 1.5 ml tubes have internal dimensions in which the pestles may not work, microtiter plates have different binding qualities, and different caseins are less efficient in blocking and may result in decreased sensitivity or higher background OD values.

1. **PBS** – phosphate buffered saline, pH ~7.4: Use stock laboratory PBS OR Dulbecco's PBS (Sigma #D5773). Adjust pH if necessary. Add 10 mg phenol red or 100 µl of phenol red stock solution (1 g/10 ml water) per 1 liter PBS. Store at 4°C. Shelf life is 2 weeks.
2. **BB** – blocking buffer: BB can be prepared in two ways (A or B). Shelf life is 1 week at 4°C; BB may be frozen. Use only ELISA grade bovine serum albumin (BSA) and casein prepared from bovine milk (Sigma C7078).

A.

BB - BSA / casein:	½ liter	1 liter
PBS, pH 7.4	500 ml	1 liter
BSA (1.0%)	5.0 g	10.0 g
casein (0.5%)	2.5 g	5.0 g
phenol red[1]	100 µl	200 µl

[1] Preparing a stock solution of phenol red (1 g / 10 ml water) eliminates the need to weigh small amounts.

1. Suspend bovine serum albumin (BSA) and casein in PBS and mix for 2 hours or until dissolved. Some casein may not dissolve.
2. Add the phenol red.

B.

Boiled casein (BB):	½ liter	1 liter
PBS, pH 7.4	450 ml	900 ml
casein	2.5 g	5.0 g
0.1 N NaOH	50 ml	100 ml
phenol red[1]	100 µl	200 µl

1. Suspend casein in 0.1 N NaOH and bring to a boil.
2. After casein is dissolved, slowly add the PBS, allow to cool, adjust the pH to ~7.4 with HCl, and add the phenol red.

BB:IG-630 – blocking buffer with IGEPAL CA-630: This is the mosquito grinding solution.

to 1 ml BB add 5 µl IGEPAL[2] CA-630

5 ml BB add 25 µl IGEPAL CA-630

Mix well to dissolve the IG-630 in the BB. Shelf life at 4°C is 1 week.

PBS:Tw – wash solution: PBS plus 0.05% Tween 20. Add 0.5 ml Tween 20 to 1 liter of PBS. MIX WELL. Store at 4°C. Shelf life is 2 weeks.

MAb stock – Monoclonal antibody stock solution: Dissolve the lyophilized MAb in diluent (1:1 distilled water and glycerol) to give stock solutions of 0.5 mg / ml (0.5 µg / µl). The water:glycerol solution prevents freezing during routine storage at -20°C.

P. falciparum = 0.20 µg / 50 µl PBS

P. vivax-210 = 0.025 µg / 50 µl PBS[3]

P. vivax-247 = 0.025 µg / 50 µl PBS[3]

[2] IGEPAL CA-630 (Sigma I3021) replaces NONIDET P-40 which is no longer available from Sigma-Aldrich. If available, NONIDET P-40 can be used.)

[3] Mabs for *P. vivax* will be made available in this kit at a future date.

Capture MAbs:

MAb	µg / 5 ml	µl MAb stock / 5 ml PBS
Pf	20 µg	40 µl stock
Pv-210	2.5 µg	5 µl stock
Pv-247	2.5 µg	5 µl stock

Peroxidase conjugated MAbs:

MAb	µg / 5 ml	µl MAb stock / 5 ml BB
Pf	5.0 µg	10 µl stock
Pv-210	5.0 µg	10 µl stock
Pv-247	5.0 µg	10 µl stock

Positive controls

The following table describes the volumes and amounts necessary for the dilution series from the stock (Vial I) starting with the lyophilized positive control. Add the volume of BB listed for Tube I and mix until the lyophilized positive control is dissolved. Using the volumes of BB recommended, perform 2 serial dilutions with thorough mixing between steps. Tube III is used as your single positive control in your initial test. If needed, further dilutions will be performed on the plate during confirmational testing.

Dissolved control solutions should be frozen for later use.

	Tube	vol. BB	Pf	conc. Pf
P. falciparum	I (stock)	200 µl +	5 µg =	25 ng / µl
	II	992 µl +	8 µl tube I =	0.2 ng / µl
	III	990 µl +	10 µl tube II =	2.0 pg / µl
	Tube	vol. BB	Pv0210	conc. Pv-210
P. vivax-210	I (stock)	200 µl +	5 µg =	25 ng / µl
	II	990 µl +	10 µl tube I =	250 pg / µl
	III	996.8µl +	3.2 µl tube II =	0.8 pg / µl
	Tube	vol. BB	Pv247	conc. Pv-247
P. vivax-247	I (stock)	200 µl +	880 µg =	4.4 µg / µl
	II	975µl +	25 µl tube I =	110 ng / µl
	III	990 µl +	10 µl tube II =	1.1 ng / µl

Mosquito Sample Preparation

1. Place the mosquito[4] in a labeled 1.5 ml micro centrifuge tube and grind in 50 µl BB:IG-630.
2. Rinse pestle with two 100 µl volumes of BB, catching the rinses in the tube (Total vol. = 250 µl).[5]
3. Before grinding the next mosquito, rinse pestle in PBS-Tw twice; dry with tissue to prevent contamination.
4. Samples may be used immediately or frozen for later analysis.

Preparation of Negative Controls

At least 7 or 8 laboratory reared female mosquitoes that are known to be uninfected[6] (same as test species if possible) should be prepared in the same way as the test samples. Use these in the same way as the test sample instructions in negative control wells. Be sure that you use a clean pestle so that contamination with a positive sample does not occur.

Developing Standard Curves

A negative cut-off value below which sporozoites will not be considered present must be determined by analysis of negative controls. Calculate the mean OD of at least 7 negative individuals after the ELISA procedure. If any has a value two times higher than the mean absorbance, retest the sample. That is, in order to determine if a sample is positive, negative, or questionable, multiple negative controls should be run in the plate with the samples. After the ELISA is completed and the OD's have been determined by the plate reader, take the average of the OD's for those negative controls. Multiply the average by 2. This is the "cutoff" value. If your sample's OD is greater than this cutoff value, they are considered positive. If sample's OD is below this cutoff value, they are considered negative. If your sample's OD is close in value to the cutoff value, run the sample in the confirmation test.

For each *Plasmodium* species, a standard positive control curve should be developed. In a microtiter plate, add 50 µl BB to wells in a column. Perform a 2 X dilution series by adding 50 µl from the appropriate Tube III to row A. Mix and transfer 50 µl to the next row well. Continue to the bottom of the plate. Perform the dilution series in triplicate. If desired, plots of the absorbance of these standards can be plotted as follows.

[4] You may grind one mosquito, one head + thorax only, or up to five pooled mosquitoes in each tube in 50 µl of BB:IG-630.

[5] The total resulting grinding volume is ideally 200 µl because CSP will be more concentrated and therefore the test more sensitive. However, each test requires 50 µl. If both an initial and confirmational test are needed, then 100 µl will be needed for each CSP. If testing for Pf, Pv210, & Pv247, then 300 µl could be needed. The likelihood of this for happening for all three is low, so 250 µl would be enough. If only testing for 1 CSP (like Pf) 200 µl will be enough and more ideal than 250 µl.

[6] This is ideal, but if not available, one can use male mosquitoes, or, if not those, then blocking buffer.

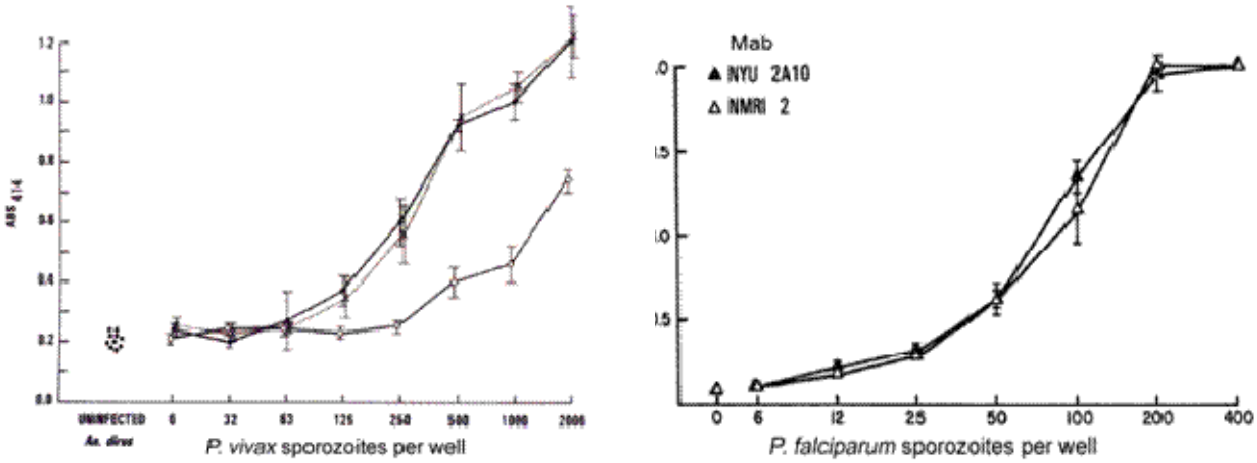


Figure 3.3.2. Examples of standardization curves developed for *P. vivax* (left) and *P. falciparum* (right). Note that near an OD of 2, the *P. falciparum* curves are not linear and would prevent correct estimates of the number of sporozoites present.

Sporozoite ELISA Directions

1. Place 50 µl of capture Mab solution in each well of the ELISA plate[7] and incubate for at least 30 min or as long as overnight at room temperature. (During this incubation, mosquito test samples and negative controls may be prepared. Alternatively, prepare the samples prior to analysis and freeze.)
2. Use a separate plate for each sporozoite species.
3. Cover plate with another plate or lid during all incubations to prevent evaporation.
4. Remove well contents by aspiration[8] and fill wells with BB.
5. Incubate 1 hour. (Alternative mosquito test sample preparation interval.)
6. Drain well contents by aspiration or banging plates sharply and add 50 µl mosquito homogenate per well.
7. Add 50 µl positive and negative control solutions to wells.
8. Incubate 2 hours at room temperature.
    a. Near the end of the incubation period prepare the ABTS - Substrate solution - This solution should be prepared fresh. Mix Solution A (ABTS from Kirkegaard Perry, www.kpl.com) and Solution B (hydrogen peroxide) 1:1. Prepare enough to add 100 µl / well. If you are doing a full plate, prepare 10 ml (5 ml of Solution A and 5 ml of Solution B).
    b. Near the end of the incubation period, mix Mab-peroxidase Conjugate in blocking buffer: 0.05 µg / 50 µl BB. Prepare enough for 50 µl / well. Hereafter we will refer to this simply as 'conjugate.'
    c. In a 1.5 ml microfuge tube, **confirm enzyme activity** by mixing 5 µl of the conjugate above with 100 µl ABTS. There should be a rapid color change indicating that the peroxidase enzyme and the substrate are functional.

[7] To fill each of the 96 wells on a plate with 50 µl requires 4.8 ml. It is convenient to make up 5.0 ml of each Mab solution and 10.0 ml of substrate (100 µl/well) per plate

[8] If a vacuum aspiration system is not available, trays should be held firmly by their sides, inverted over a sink or tray, and banged gently to remove the solutions. Follow this by blotting on clean absorbent paper.

9. Remove homogenate.
10. Wash wells 2 X with PBS-Tw by filling and emptying the wells.[9]
11. Add 50 µl conjugate to each well
12. Incubate for 1 hour
13. Remove conjugate
14. Wash wells 3 X with PBS-Tw.
15. Add 100 µl ABTS per well.
16. Incubate for 30 and / or 60 min.
17. Read visually, or determine OD at 405-414 nm[10] at both 30 and 60 minutes.

References

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9 If background has been observed in previous analyses, a third wash may be performed.

10 Absorbance values of OD > ~ 2 are in the non-linear portion of the standard curve. If quantitative information is required, dilute the strong positives 1:1 by adding 150 µl BB before retesting (Original grinding volume was 200 µl, minus 50 µl removed for testing). Repeat if necessary to insure that the absorbance value for the test sample is in the linear portion of the positive control curve (0.5 to 1.5 OD units) before calculating the number of sporozoites.

SPOROZOITE ELISA NO:_____

(Sample analysis)

DATES: Coat:_____ Test:_____

	1	2	3	4	5	6	7	8	9	10	11	12
A	pos	neg										
B												
C												
D												
E												
F												
G												
H												

SPOROZOITE ELISA NO:_____

(Standard Curves or 'Confirmation test')

DATES: Coat:_____ Test:_____

			+ control dilutions rep 1	rep 2	rep 3							
	1	2	3	4	5	6	7	8	9	10	11	12
A	neg 1		2 X	2 X	2 X							
B	neg 2		4 X	4 X	4 X							
C	neg 3		8 X	8 X	8 X							
D	neg 4		16 X	16 X	16 X							
E	neg 5		32 X	32 X	32 X							
F	neg 6		64 X	64 X	64 X							
G	neg 7		128 X	128 X	128 X							
H	neg 8		256 X	256 X	256 X							